TOWARD POSITIONAL CLONING OF EVERBLOOMING GENE (*EVB*) IN PLANTS: A BAC LIBRARY OF *ROSA CHINENSIS* CV. OLD BLUSH

A Thesis

by

GREGORY HESS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Plant Breeding

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Approved by:

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Committee Members,	David Byrne
	Dirk Hayes
Head of Department,	C. Wayne Smith

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ABSTRACT

Toward Positional Cloning of Everblooming Gene (*evb*) in Plants: A BAC Library of *Rosa chinensis* cv. Old Blush. (August 2005)

Gregory Hess, B.S., University of Texas at Arlington

Chair of Advisory Committee: Dr. Hongbin Zhang

A majority of commercial rose varieties bloom repeatedly throughout the year, as compared to most rose species, other woody ornamentals, and fruit crops that bloom once a year. This recurrent flowering feature of the commercial roses resulted from a flowering mutation named everblooming (*evb*). The mutation is recessive to once blooming and is found in the rose species *Rosa chinensis*. Although several molecular maps have been developed for rose, little is known about the *evb* gene, except for its classic genetics. The purpose of this study was to develop a large-insert bacterial artificial chromosome (BAC) library as a starting tool for molecular cloning and analysis of the *evb* gene by map-based cloning. To construct the large-insert BAC library, nuclear megabase-size DNA was isolated from the recurrent blooming diploid species, *Rosa chinensis* cv. Old Blush. The DNA was then partially digested with *Bam*HI and separated on agarose gels by multi-phase pulsed-field gel electrophoresis. Size selected fragments estimated between 100 kb and 150 kb in size were cloned into the pECBAC1 BAC vector and the clones having rose DNA inserts were arrayed in 80 384-well microplates individually, with each clone being bar-coded. The library contains 30,720 clones, has an average insert size of 108 kb and covers roughly 5.9x genome equivalents, with a >99% probability of isolating a single-copy clone

from the library. The library is now available to be screened with the genes cloned from other species that control vernalization and floral development and will be used in map-based cloning of the *evb* gene using a *Rosa wichuraiana* ('Basye's Thornless') x 'Old Blush' backcross population.

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INTRODUCTION

Roses (*Rosa* species) are considered to be one of the most economically important ornamental crops. They are used for garden and cut flowers (Gudin 1998) and are harvested for extraction of their various compounds for perfume and medicinal uses. Roses have been cultivated as ornamental crops for thousands of years, yet only from the early 20th century have breeders used the knowledge of genetics in rose breeding and genetic improvement (DeVries and Duboise 1984,1996). There are between 170 and 300 species of roses with various ploidy levels, but only 8 -10 of them have been used in ornamental culture (Ma 2000; Rajapakse et al. 2001). Historically, the tetraploid or triploid hybrid roses have been cultivated for floral fragrance, quality, color, and blooming characteristics, whereas some wild species have been found recently to exhibit agriculturally relevant resistance to black spot and powdery mildew (Malek and Debener 1998; Linde and Debener 2003). In addition, other traits such as winter hardiness, male sterility, and absence of prickles have also been studied genetically (Semeniuk 1971; DeVries and Dubois 1978, 1984; Malek and Debener 1998; Debener 1999).

Roses have relatively small genomes. It has been estimated that the genome sizes of roses are between 380 and 670 megabases (Mb)/1C. (Yokoya et al. 2000; Rajapakse 2001). Being only 4 times the size of *Arabidopsis*, the relatively small genome size of rose facilitates the isolation of genes that are unique in the species through positional or map-based cloning strategies. Nevertheless, it is currently not possible to clone the genes of

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This thesis follows the style of Theoretical and Applied Genetics.

interest in the species due to a shortage of essential resources and tools such as a large-insert DNA library essential for the process of isolating genes known by phenotype for positional cloning.

In addition to their relatively small genome size, roses have many interesting and important traits that are unique or absent in the model plant species, such as *Arabidopsis thaliana*, rice, *Medicago truncatula,* and *Prunus persica*. One of the most significant is the gene controlling all season blooming (Semeniuk 1971; DeVries and Dubois 1984). This gene is designated as the recurrent flowering or ever-blooming (*evb*) gene. Recurrent flowering refers to a plant's ability to bloom repeatedly throughout the year. The rose *evb* gene is a unique single gene mutation discovered so far within the plant kingdom controlling recurrent flowering. More importantly, it has been shown to be conditioned by a recessive allele at a single locus (Semeniuk 1971; DeVris and Dubois 1978, 1984, 1996; Debner et al. 2001). Normally, seasonal blossoms begin after a 1 - 3 year plant juvenility or maturation stage has occurred. Vernalization provided by winter cold often is required for transition from vegetative growth to reproductive inflorescence (Taiz and Zeiger 1998) and is possibly mediated by epigenetic actions or alternatively splicing related genes (Koornneef et al. 1994; Lee et al. 1994; Chandler et al. 1996; Gendall et al. 2001). Instead of having a single bloom period in spring, the *evb* mutant confers recurrent flowering throughout the entire growing season (Semeniuk 1971; DeVries and Dubois 1984, 1996). Moreover, the mutant has also been found to significantly reduce the juvenility period to first bloom. Therefore, the mutant not only enables earlier reproduction of plants by a few years, but also ensures continued production, even after an unexpected spring frost that kills the first set of blooms. As advances in biotechnology have enabled more efficient gene

transfer in many crop plants, the *evb* gene, once cloned, could be translated (Gould et al. 2002; Condliffe et al. 2003) into more precocious and extended harvest or bloom period in other woody ornamentals, small fruit, and fruit tree species.

Because there is no information on the sequence and biochemistry of the *evb* gene, the positional or map-based cloning approach has emerged as the method of choice to clone the gene. There are three major prerequisites to clone a gene of interest by positional cloning. One is the genetic mapping of the target gene to its locus in the genome using DNA markers. The second one is a large-insert DNA library that is required to approach the gene from its closely linked DNA marker(s) via chromosome walking. The third one is the availability of genetic transformation technique for gene identification through genetic complementation. The recent development of DNA markers (AFLPs, RFLPs and SSRs) and genetic maps in roses have allowed genetic mapping of various traits (Debener 1999; Rajapakse et al. 2001; Crespel et al. 2002). Additionally, agrobacterium-mediated transformation has demonstrated the ability to introduce genes of interest into the rose (Firoozabady et al. 1994; van der Salm et al. 1997). These tools, combined with the recent development of a *Rosa wichuraiana* ('Basye's Thornless') x 'Old Blush' backcross population (WOB; unpublished), will allow mapped-based cloning of the *evb* gene and its subsequent introduction into once blooming trait plants. As such, the availability of a large-insert DNA library for the rose line that harbors the *evb* gene is the limiting factor of isolating the gene.

Large-insert bacterial artificial chromosome (BAC) libraries have been proven in many crop plants to be desirable resources for genome research, including positional gene cloning and characterization. Maintaining a relatively large insert size and high stability

while having low level of chimeric clones and ease of purifying cloned DNA makes the BAC system the prevailing choice for complex genome analysis (Zhang et al. 1996). Therefore, to facilitate positional cloning of the black spot resistance gene (*Rdr1*), one of the most serious fungal diseases in roses, Kaufmann et al. (2003) previously constructed a BAC library from a tetraploid clone of *Rosa rugosa*. Using this BAC library, they are working toward isolation of the *Rdr1* gene by positional cloning (Kaufmann et al. 2003). This BAC library consists of 27,264 clones and has an average insert size of about 102 kb, being equivalent to about 5.2 x of the *R. rugosa* haploid genome. Because our *long-term goal* of this research is to isolate the *evb* gene by positional cloning, the objective of this study was to construct an essential large-insert BAC library from a rose line containing the target *evb* gene.

MATERIALS AND METHODS

Plant Materials and HMW DNA Isolation

Green house-grown diploid *R. chinensis* cv. 'Old Blush' plants ($2x = 2n = 14$) were pruned significantly to allow for new shoot development and then immediately fertilized with water soluble Technigro-Plus (20 N-18 P-18 K) by dissolving 1oz. into 1 gallon of water. After the new shoots appeared at about 5 days post fertilization, the rose plants were placed in the dark for 4 days to minimize the phenolic and other metabolic substances that potentially affect DNA quality. Between 20-50 grams of young leaf and shoot tissues were then harvested from the new growth and either frozen for storage at –80^{o}C, or ground in liquid nitrogen to isolate nuclei according to Zhang et al.. (Zhang et al. 1995; Zhang 2000), with modifications by increasing the number of centrifugation washes interspersed with subsequent filtrations by clean Mira-cloth layered in new cheesecloth.

Homogenization of tissue was achieved by grinding approximately 50g of frozen or fresh young leaf tissue into fine powder in liquid nitrogen with mortar and pestle, then immediately transferred into an ice cold beaker containing 1 L ice-cold 1x nuclei extraction buffer [0.5 M sucrose, 10 mM Trizma, 80 mM KCl, 10 mM EDTA, 1 mM spermidine (spd), 1 mM spermine, pH 9.4-9.5, 0.15% (v/v) β-mercaptoethanol, 0.5% (v/v) Triton X-100]. The mixture was incubated on ice for 10 min with gentle stirring, and then filtered with one layer of Mira-cloth and two layers of cheesecloth into six ice-cold 250 ml-centrifuge bottles. Since the homogenate was sticky, it was filtered with assistance by handy squeezing. The nuclei in the filtrate were pelleted by centrifugation in a fixed-angle rotor at 1,880 g at 4^{o}C for 20 min. After discarding the supernatant, re-suspension of the pellet was

achieved by adding 1ml of the nuclei extraction buffer while assisting re-suspension by agitating with a small paintbrush; and then adding additional 30 ml of the nuclei extraction buffer. The nuclei suspension was filtered again with two layers of Mira-cloth into a 50 ml centrifuge tube and pelleted in a swinging bucket centrifuge at 1,920 g (note that g is not equal to rpm) at 4^{o}C for 20 min. In the same way, the resulting pellet was washed and filtered three more times. After the final wash, the nuclei were re-suspended in 1-5 ml of the nuclei extraction buffer without B-mercaptoethanol and Triton X-100, diluted to approximately 5 x 10^{7} nuclei / ml, and then stored on ice.

Embedding the Nuclei in Low-melting-point (LMP) Agarose Plugs and Purification of Megabase DNA

After obtaining a satisfactory quality and concentration of *R. chinensis* nuclei (Zhang et al. 1995), the nuclei were then pre-warmed at 45^{o}C in a water bath for 5 min, mixed with a same volume of melted 1% LMP agarose maintained in the 45^{o}C water bath and aliquoted into 100-ul moldings on ice with a cut-off pipette tip. After completely solidifying, the agarose plugs containing rose nuclei were transferred into 5-10 volumes of the nuclei lysis buffer containing 0.5 M EDTA, pH 9-9.3, 1% sodium lauryl sarcosine, and 0.3 mg/ml proteinase K and incubated at 50^{o}C for 24 hours with gentle rotation shaking (Zhang et al. 1995). The plugs were then washed once in ice-cold TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0), three times in ice-cold TE plus 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), and three times ice-cold TE, with one hour each wash. The plugs were stored in TE at 4^{o}C before use.

Determination of Optimal Partial Digestion Conditions

To determine optimal conditions for partial digestion of DNA embedded in the LMP agarose plugs, reactions were set up according to Zhang (2000). Specifically, three 100-µl plugs were cut into equal slices, 9 slices/plug, with a glass slide cover and incubated with 8.73 ml of buffer I (7,803 µl H_2O, 900 µl 10 x enzyme reaction buffer, 18 µl 1 M spermidine, 9 µl 1 M DTT) on ice for 30 min. The buffer was then drained, replaced with the same volume of fresh Buffer I and incubated on ice for an additional 30 min. The buffer was discarded again, and the gel slices containing rose DNA were transferred into 1.5 µl micro-tubes, with three slices per tube and each tube containing 170 µl of Buffer II (142 µl H_2O, 17 µl 10 x enzyme reaction buffer, 0.34 µl 1M spermidine, 0.17 µl 1 M DTT, 10 µl 10 mg/ml BSA). The restriction enzyme *Bam*HI was added to each tube at the following amount of the enzyme: 0.0, 0.1, 0.25, 0.5, 0.8, 1.0, 1.2, 2.0, or 4.0 units. The reactions were incubated on ice for 100 min, and then transferred d to a 37^{o}C water bath and incubated for 8 min. The reactions were stopped by immediately transferring the tubes after the 8-minute incubation onto ice and adding 1/10 volume of 0.5 M EDTA, pH 8.0.

The partially digested rose DNA in the agarose plug slices were loaded into a 1% pulsed-field agarose gel in 0.5 x TBE (45 mm Truism base, 45 mm boric acid, 1 mm EDTA, pH 8.3), and sealed with the agarose used to make the gel. The λ DNA ladders were also loaded along with the rose DNA as DNA molecular weight markers. The pulsed-field gel electrophoresis (PFGE) was performed 0.5 x TBE under the following condition: 12.5^{o}C (cooler settings), 80 (pump settings), 120^{o} angle, 6 V/cm, initial pulse time of 50 seconds, and final pulse time of 50 seconds for 20 h. The gel was then stained with ethidium bromide and photographed.

Large-scale Partial Digestion of Megabase DNA, Size Selection, Ligation and Transformation

After optimal conditions have been determined, a large-scale partial digestion of 10 100-µl plugs was performed using the optimal enzyme concentration determined above according to Zhang (2000) with some modifications. Instead of individual comb wells, a gel was prepared with a single long trough to hold the partially digested DNA plug slices for size selection by PFGE. The PFGE was conducted at 12.5^{o}C (cooler settings), 80 (pump settings), 120^{o} angle, 6 V/cm, initial pulse time of 90 seconds, and final pulse time of 90 seconds for 14 h, followed by 12.5^{o}C (cooler settings), 80 (pump settings), 120^{o} angle, 4 V/cm, initial pulse time of 5 seconds, and final pulse time of 5 seconds for 5 h., The partially digested DNA with a size range from 100 – 400 kb was collected. As described by Zhang (2000), the DNA marker lane and 2-3 mm of the DNA sample trough were excised, stained, photographed, and re-positioned to the gel to locate the zone of gel ranging from 100-400 kb. The excised gel zone containing the target DNA fragments was divided into three 0.5-cm sections representing fragments ranging from 100-200 kb, 200-300 kb, and 300-400 kb, respectively. These 0.5-cm thick gel strips were then electroeluted in dialysis tubes (Zhang 2000). The three separate sized fractions were quantified by gel electrophoresis using λ DNA standards of known concentration as the standards, and ligated into the *Bam*HI-digested, dephosphorylated BAC vector pECBAC1 at a molar ratio of 4:1 with an excess amount of vector (Zhang et al. 1996).

Transformation and Screening of Ligations

After the ligations for fractions 1,2, and 3 were made, 1 µl of each ligation reaction was gently mixed with 20 µl of transformation competent *E. coli* DH10B cells (Invitrogen) and transformed into the competent cells by electroporation using a Life Technology Cell Porator at the settings: 350V, 330 uF, low ohms, fast charge rate, and 4 K ohms. The transformed cells were cultured in 1 ml of SOC medium (Invitrogen) at 200 rpm, 37^{o}C for 1 h. Each fraction ligation culture was plated on the selective agar medium containing 32 g LB agar, 1 ml 12.5 mg/ml chloramphenicol, 75 ul 200 mg/ml IPTG and 3 ml 20 mg/ml X-gal per liter, and grown for a minimum of 24 h in a 37^{o}C incubator for colony color development.

White bacterial colonies presumably containing the rose DNA inserts from each of the three plates (fractions 1, 2, and 3) were analyzed according to Zhang (2000) to estimate the insert sizes of the clones of each ligation. Ten colonies were randomly selected from each of Fractions 1, 2 and 3, respectively and grown at 37 ^{o}C, 250 RPM overnight, each in 5 ml of Lure Broth (LB) containing 5 µl 12.5 µg/ml chloramphenicol (CM). The 30 overnight cultures were then centrifuged for 10 min at 3,200 RPM, and the cell pallet was decanted and re-suspended in the remaining medium (about 0.20 ml) by Vortexing. Two hundred microliters of Solution 1 (50 mM glucose, 10 mM EDTA, pH 8.0) was added to each of the 30 cultures and incubated on ice for 5 min After the incubation, 400 µl of freshly made Solution 2 [0.2 N NaOH, 1% (w/v) SDS] was added to each culture and mixed gently by rotating the culture tubes on long axis several times. Three hundred microliters of Solution 3 (3.0 M potassium acetate, pH 5.2) was then added to each of the tubes, mixed gently and incubated on ice for 15 - 30 min. To separate the BAC DNA from the bacterial host

chromosome DNA, the precipitate was centrifuged at 4°C, 3,200 RPM for 15 min. From each tube, 750 µl of the supernatant was transferred into a new 1.5-ml microtube. To the supernatant, 450 µl isopropanol was added mixed and centrifuged at room temperature, 12,000g for 5 min. The supernatant was discarded, and the DNA pellet was washed in 70% ethanol, air-dried for about 29 min and re-suspended in TE.

BAC Insert Analysis by PFGE

10 µl from each of the 40 µl DNA isolate solutions was digested with 3 U *Not* I for 3 hours at 37°C in a reaction volume prepared on ice of 40 ul including 23.7 µl H_2O, 4 µl 10 x *Not* I buffer, 2 µl 40 mM spermidine. The reactions were stopped by adding 1/10 volume (4 µl) of 10 x loading dye, then heated the at 65°C for 10 minute to separate sticky ends, immediately plunged into ice, and run on a 1% agarose by pulsed-field gel electrophoresis in 0.5 x TBE under the following condition: 12.5°C (Cooler settings), 80 (Pump settings), initial pulse time 5 seconds, final pulse time 15 seconds, 120 degree, 6V/cm, and 16 hours. The gel was stained for 30 minutes, destained for 30 minutes in water, and photographed under UV light.

Library Assembly

After selecting the fraction ligation(s) that yielded clones with satisfactory insert sizes, a large-scale transformation was performed on aliquoted vials of 40 µl rose DNA insert/vector into 2 µl increments as described above. White colonies were hand picked into 384-well microplates, and incubated overnight at 37 °C in freezing media as described by Zhang (2000). A total of 80 384-well microplates were assembled for the library. To

facilitate its long-term storage and screening, the library was duplicated into two copies, with one copy for long-term storage for use of rose genome research and the other one for routine library screening.

High-density Clone Filter Preparation for Library Screening

High-density clone filters were prepared according to Zhang (2000) by use of the Genomic Solutions GeneTAC G3 Robotic Workstation. Four 384-well microplates were double-spotted onto 7.5 x 11.5 cm filters (+ an extra copy) in a '3 x 3' fashion. superimposed on LB agar medium containing 12.5 μl/ml chloramphenicol and incubated at 37^{o}C overnight. Subsequent filter preparation and fixation of BAC DNA to the membranes was according to the procedure described by Zhang (2000).

Primer Design

To characterize the library and facilitate isolation of the *evb* gene, we searched the GenBank for homeologous vernalization and flowering genes cloned to date in other plant species (Zemetra and Morris 1988; Schultz 1991; Mandel et al. 1992; Lee et al. 1994; Chandler 1996; Dubocovsky et al. 1998; Michaels and Amasino 1999; Johanson et al. 2000; Kitahara and Matsumoto 2000; Kotoda et al. 2000; Sheldon et al. 2000; Gendall et al. 2001; Kitahara et al. 2001; Pelaz et al. 2001; Ampomah-Dwamena et al. 2002; Murai et al. 2003; Yan et al. 2003, 2004; Caicedo et al. 2004; Pillitteri et al 2004). The gene sequences were imported into the PRIMER3 software for primer design (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). These sequences before primer design were BLASTed against each other with National Center for Biotechnology Information (NCBI) databases

(http://www.ncbi.nlm.nih.gov/BLAST/ , http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) by comparing FASTA report common sequences (Tatusova and Madden 1999). Only the unique regions of each gene were used in the primer design for each gene. The primer pairs for each gene were designed using the program "Primer3" with additional criteria to select target sequences greater than 450 kb for PCR amplification of genomic DNA (Saiki 1990; Zhang 2000). This will maximize the probability of obtaining positive clones from the library using these homeologous sequence probes.

RESULTS

Improved HMW DNA Isolation from Roses

High-molecular-weight (HMW), readily digestible and clonable DNA, is essential for large and complex genome analysis. To facilitate genome research of *Rosa* species, in particular for construction of large-insert BAC libraries, we previously attempted to isolate HMW DNA from roses according to Zhang et al. (1995) (unpublished). Although DNA fragments >800 kb were obtained and readily digestible, they were difficult to be cloned into BAC vectors due to significant contamination with polyphenolic substances, as indicated by the dark brown color of the DNA agarose plugs (Fig. 1A). We also attempted to minimize the phenolic substance contamination by adding polyvinyl pyrrolidone 40 (PVP40) to the DNA extraction buffer of Zhang et al. (1995) as Kaufmann et al. (2003), however, the resultant DNA agarose plugs remained dark brown in color, suggesting that the DNA was not readily clonable. Therefore, we attempted in this study to minimize the polyphenolics by modifying the procedure of Zhang et al. (1995) but not the buffer system (see Materials and Methods).

We particularly made two modifications on the procedure of Zhang et al. (1995). First, we controlled the rose plant metabolism by maintaining the plants in dark for a few days before tissue collection. This would significantly reduce the phenolic and other metabolic substances that potentially inhibit DNA cloning. Second, we increased wash times of the nuclei from 1 – 2 to 6 times before they were embedded into LMP agarose plugs. We modified this step because all of the major metabolic substances, including polyphenolic substances, are synthesized in cytoplasm, and the increased number of

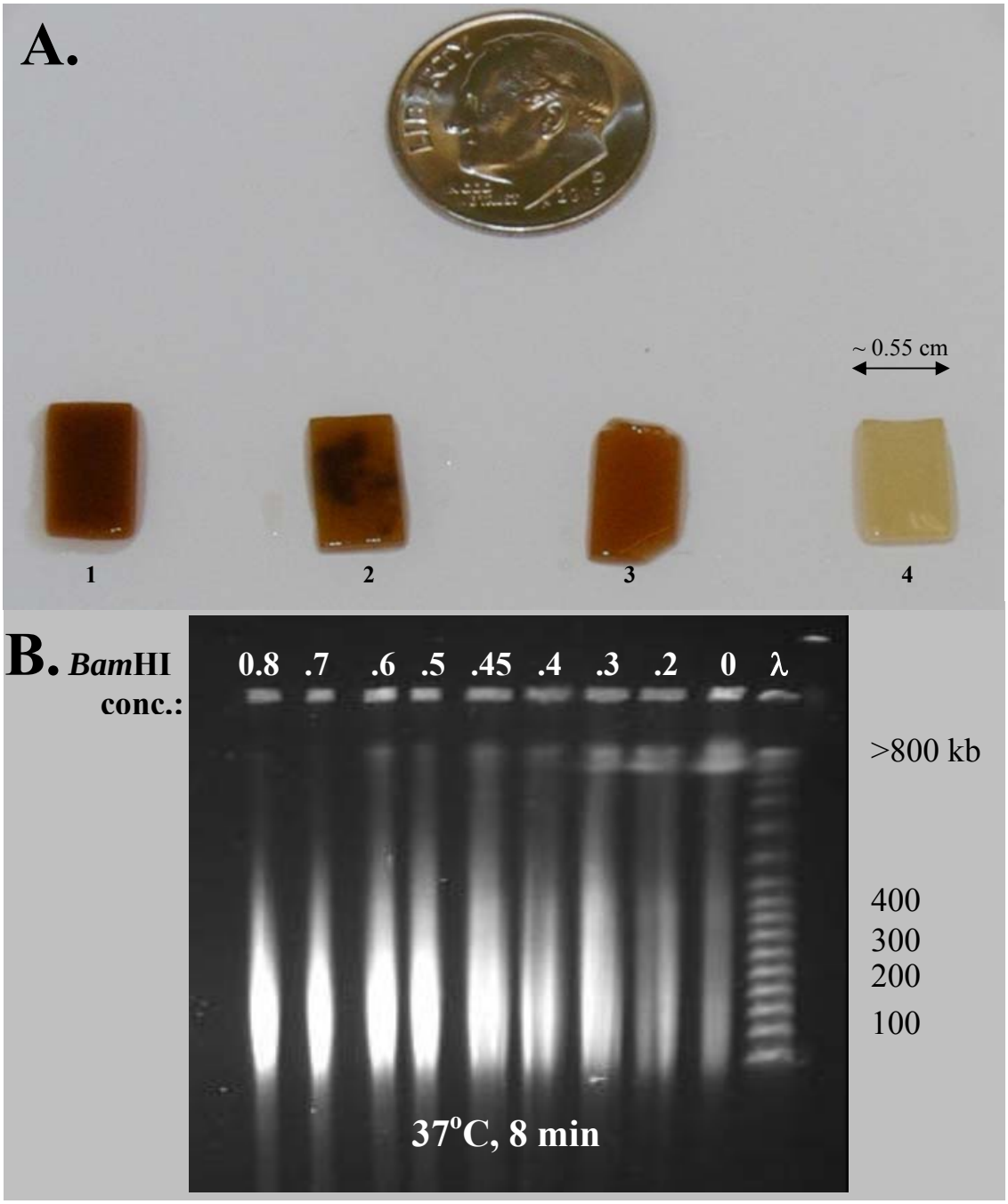

Figure 1. Megabase-sized DNA isolated from rose. A) LMP agarose plugs of the rose DNA isolated with the buffer of Zhang et al. (1995) with (1) and without (2 and 3) PVP40, and the procedure modified in this study (4). Note the differences in phenolics levels indicated by the plug color, with the darkest brown having the most phenolics. B) Rose DNA undigested (0) and partially digested with a series of concentrations of *Bam*HI. 0.6 unites of *Bam*HI (the third lane from left) was selected for large-scale partial digestion and library construction.

washing and filtrations to the nuclei would minimize the contamination of polyphenolic and other metabolic substances to the nuclei. Figure 1A shows an example of the rose cv. Old Blush DNA LMP agarose plugs isolated with the modified procedure. In comparison with the DNA plugs isolated before, the color of the rose DNA plugs was much lighter even though they were still beige in color. This result suggested that the polyphenolic substance contamination of the plugs had been significantly reduced, thus the clonability of the DNA into BAC vectors was likely increased.

To test the size, digestibility and clonability of the rose DNA, we digested the DNA with a series of concentrations of *Bam*HI in a controlled time period (37^{o}C for 8 min) and analyzed the digested DNA, along with undigested DNA, on a pulsed-field gel (Fig. 1B). For the undigested DNA, although some of the DNA fragments were observed to be < 400 kb in size on the pulsed-field gel that were not suited for large-insert cloning, a majority of the fragments were compressed in the 800-kb compression zone of the pulsed-field gel. This suggested that the DNA isolated with the modified procedure had sufficiently large size for cloning inserts of 150 kb or larger. For the digested DNA, it was observed that the degree of digestion was markedly increased as the amount of enzyme used increased, suggesting that the DNA was readily digestible for large-insert BAC cloning. Of the varying concentrations of enzyme, 0.6 units of *Bam*HI appeared to produce the greatest amount of restricted fragments arranging between 100 and300 kb, when compared to the lambda ladder DNA molecular weight standard. Therefore, the concentration of the enzyme was selected for BAC library construction.

BAC Library Construction

To answer the question whether the DNA was clonable into BAC vectors and able to construct a BAC library from the rose everblooming cv. Old Blush for positional cloning of the *evb* gene, we partially digested the DNA isolated with the modified procedure using 0.6 units of *Bam*HI per reaction under the condition determined above. The DNA was then size selected on a pulsed-field gel and the DNA fragments ranging from 100 to 400 kb was excised (Fig. 2). To enhance the cloning efficiency of suitable insert sizes, we further subdivided the gel zone containing DNA fragments of 100 – 400 kb into three fractions, with expected size of DNA fragments being 100 – 200 kb, 200 – 300 kb, and 300 – 400 kb for fractions 1, 2 and 3, respectively (Fig. 3). The DNA fragments in each fraction were electroeluted, characterized for concentration (Fig. 4), ligated into the *Bam*HI-digested, dephosphorylated pECBAC1 vector (Fig. 5) and transformed into the bacterial host (Table 1). Ten white clones were randomly selected from each ligation and analyzed by PFGE. Fractions 1, 2 and 3 gave 25, 420 and 3 white clones presumed containing rose DNA inserts, respectively (Table 2). The average insert sizes of the clones were shown to be 83, 103 and 120 kb for Fractions 1, 2 and 3. Although Fraction 3 yielded much larger-insert clones than Fractions 1 and 2, but yielding only a few clones per transformation made it difficult to construct a BAC library from such a low transformation efficiency ligation. In comparison, Fraction 2 yielded clones having reasonable average insert size (103 kb) and was also high in transformation efficiency. Therefore, we decided to construct the rose cv. Old Blush BAC library from this fraction ligation.

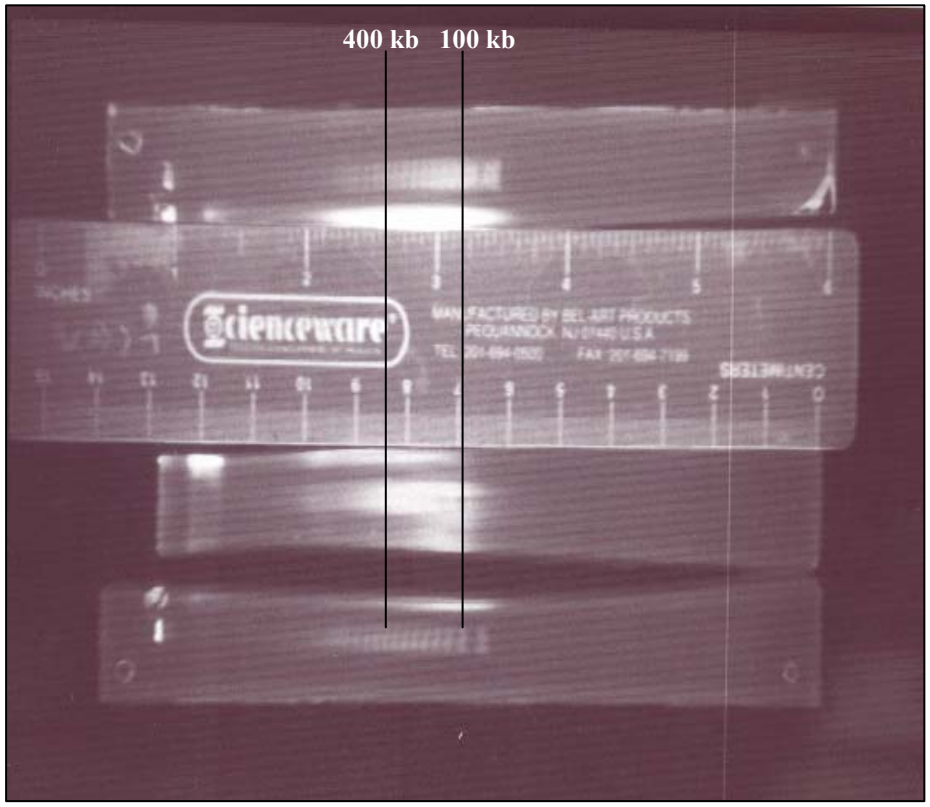

Figure 2. Rose DNA partially digested with *Bam*HI (0.6 units, 37°C for 8 min) and size-selected on a pulsed-field agarose gel. The 100 – 400 kb zone was selected for BAC library construction.

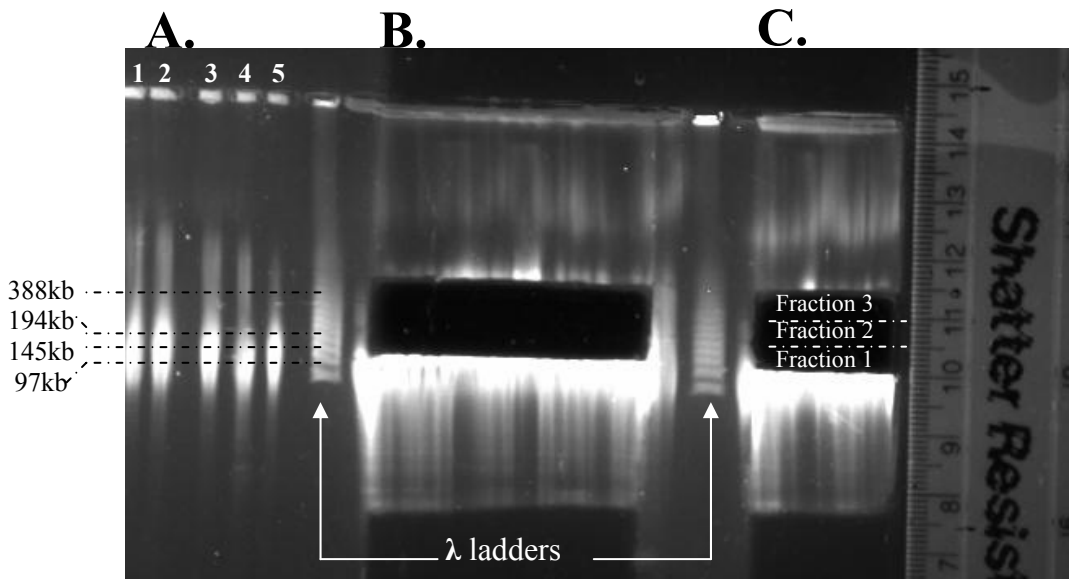

Figure 3. Size selection of *Bam*HI-partially digested fragments on a pulsed-field gel for BAC library construction. A. 3/9 of a 100-µl rose DNA plug digested with 0.6, 0.8, 1.0, 1.2, and 2.4 units of *Bam*HI (from lane 1 to 5), respectively, at 37°C for 8 min. B. Verification of the desirable-size DNA collection. The DNA fragments of 100 – 400 kb located in Fig. 2 were collected. The figure shows the remaining DNA fragments and verifies the proper collection of the desirable DNA fragments for BAC library construction. C. Factions of the selected DNA zone. Fractions 1, 2, and 3 represent the DNA migrating size of 100 – 200 kb, 200 – 300 kb, and 300 – 400 kb, respectively.

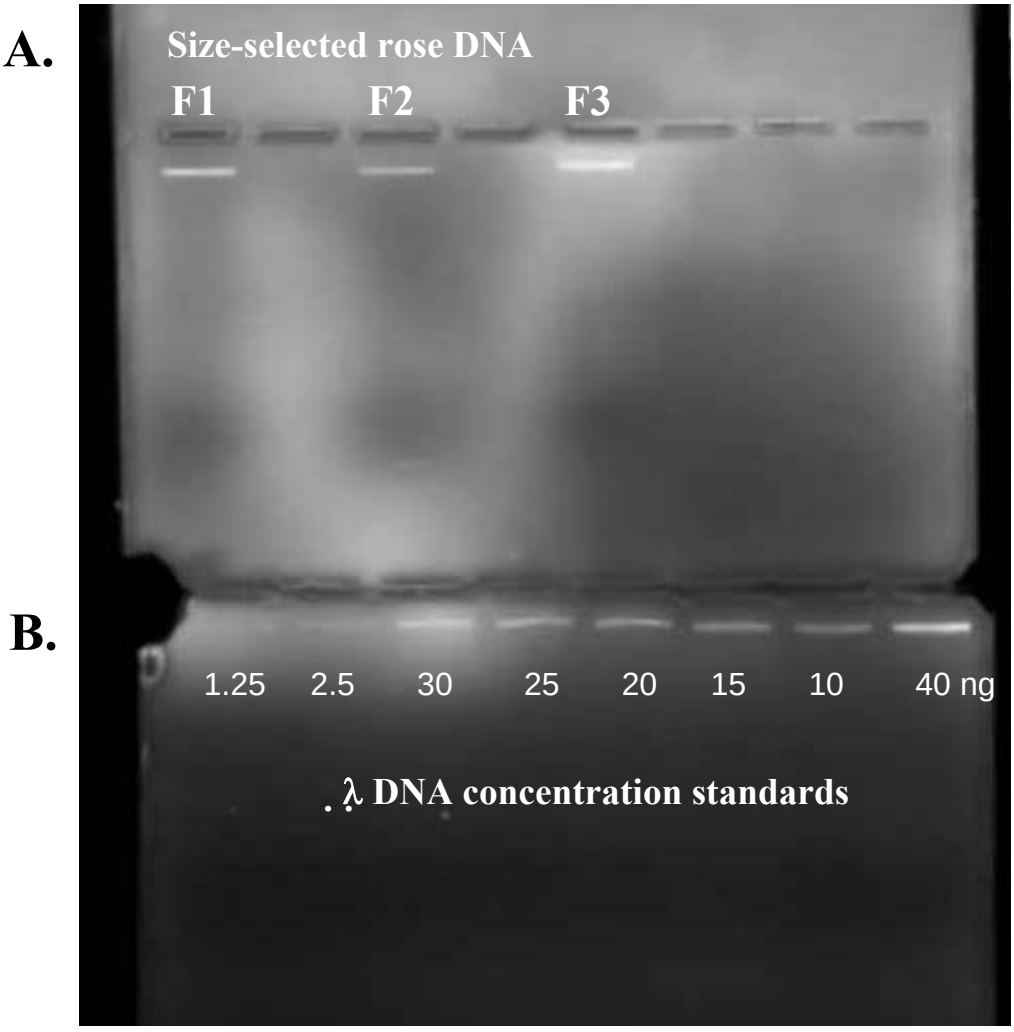

Figure 4. Quantification of DNA fragments resulted from size selection on a 1% agarose gel. A. 10 μl of the DNA electroeluted from each of fractions 1, 2, and 3. B. Lambda standards containing 1.25, 2.5, 30, 25 20, 15, 10, and 40 ng DNA per lane.

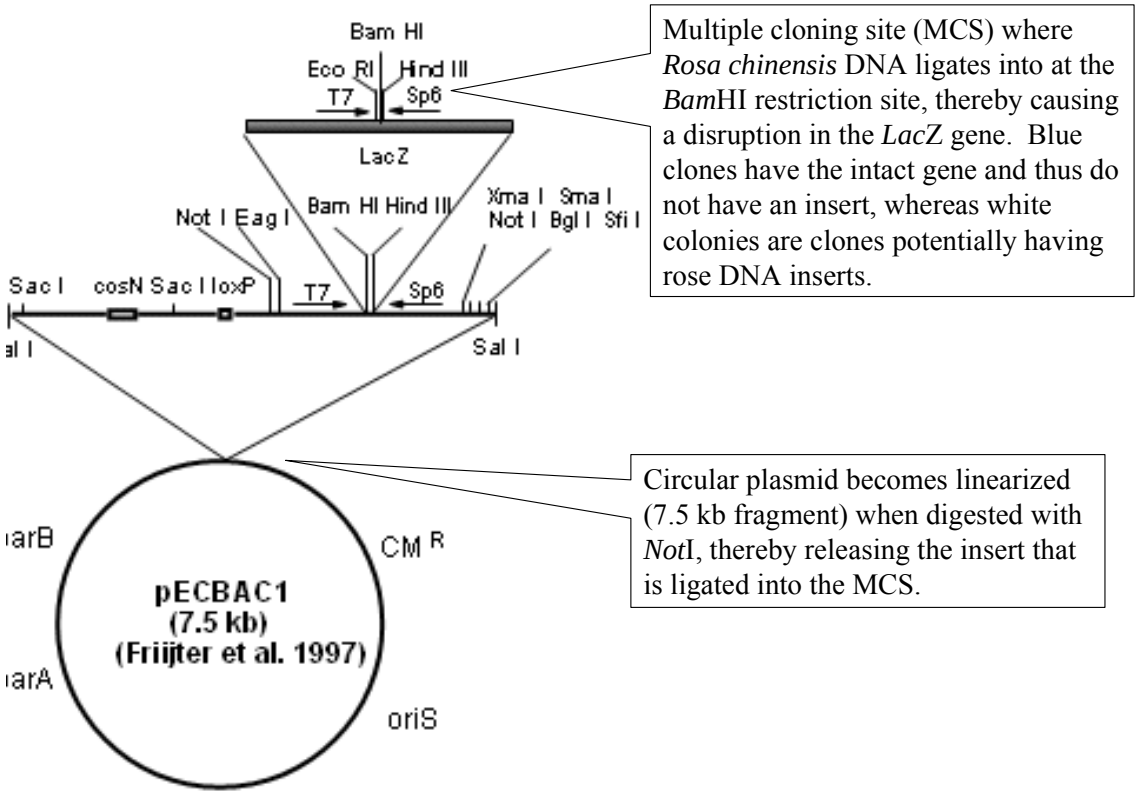

Figure 5. BAC vector pECBAC1.

Table 1. Ligation of the three fractions of size-selected DNA fragments into the *Bam*HI-digested, dephosphorylated pECBAC1 vector.

	Fraction 1	**Fraction 2**	**Fraction 3**
H_2O	60.3 ul	0	0
R. chinense DNA	120.0 ul	390.0 ul	190.0 ul
pECBAC1 vector	7.2 ul	7.8 ul	1.9 ul
5x buffer	48.0 ul	102.0 ul	50.0 ul
T4 DNA Ligase(1u/ul)	4.5 ul	10.2 ul	5.0 ul
total volume	240.0 ul	510.0 ul	246.9 ul

Table 2. Summary analysis of the clones resulting from three fractions of size selected DNA fragments.

N=10	White Colonies	Insert size Ave. (kb)	Blue Colonies	% White (w:b)
Fraction 1	31	83	155	16.7
Fraction 2	**469**	104	160	74.5
Fraction 3	05	131	43	10.4

A total of 30,720 clones were individually arrayed into 80 384-microtiter microplates for the rose cv. 'Old Blush' BAC library. To more accurately estimate the average insert fragment lengths of the library, an additional 131 clones were randomly selected and cultured overnight. BAC DNA was isolated, digested with *Not*I to release the rose DNA inserts from the BAC cloning vector and run on a pulsed field gel (Fig. 6). The insert size of each clone was calculated by adding all bands derived from the rose DNA insert of the clone. The insert sizes of the 140 clones ranged from 40 to235 kb with an average insert length of 108 kb, with approximately 50% of the clones being > 100 kb (Fig. 7). Less than 3% of the selected clones had no insert. If 2% of the clones were assumed to be derived from chloroplasts (Tao et al. 2002; Chang et al. 2003), at least 29,194 clones were derived from the nuclear DNA of rose. Therefore, the library has a conservative estimate of a 5.6 x genome coverage, based on a genome size of 560 Mb/1C for the rose species. The probability of obtaining a positive clone from the library using any single-copy sequence is greater than 99% and thus the library could be useful for positional cloning of the *evb* gene and other rose genome research. To facilitate the long-term maintenance and use of the library, we have duplicated it into two copies, with one of them for long-term storage and the other for library screening.

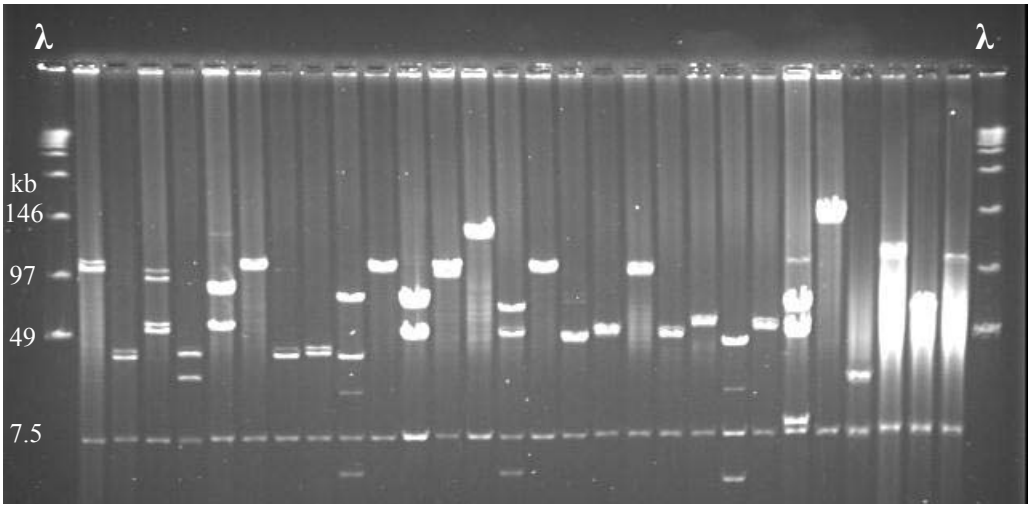

Figure 6. *Rosa chinensis* BACs digested with *Not* I and fractioned by PFGE. BAC DNA was isolated, digested with *Not* I to release the rose DNA inserts from the BAC vector (7.5 kb) and run on a pulsed-field gel. Lanes 1 and 30 indicate lambda DNA ladder whereas the remaining lanes indicate the BACs randomly selected from the rose cv. Old Brush BAC library. The insert size of each BAC was estimated by summing up the sizes of rose insert bands in the BAC lane. Note that some of bands were derived from multiple fragments that had the same or similar migration rate (or size), as indicated by significantly higher density of the bands relative to their neighboring bands on the same BAC lane.

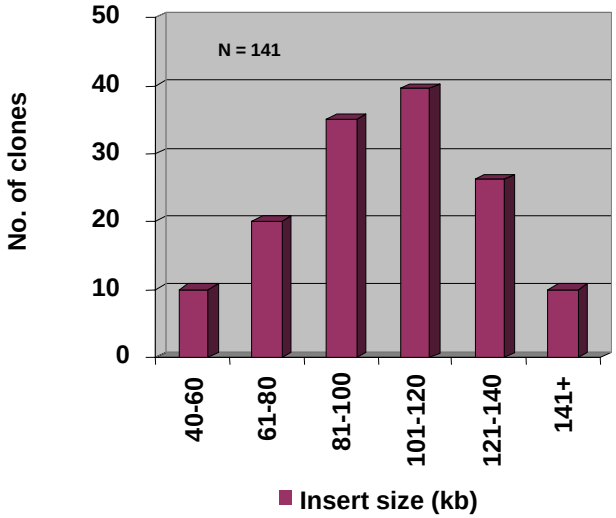

Figure 7. Insert size distribution of the *R. chinensis* cv. Old Blush BAC library.

Library Filter Preparation and Gene-specific Primer Design for Isolation of the BAC Clones Containing Candidate Genes for Rose Everblooming (*evb*)

There are several genes involved in plant flowering cloned from a variety of species including Arabidopsis, Brassica, rose, tomato, and wheat (Zemetra and Morris 1988; Schultz 1991; Mandel et al. 1992; Lee et al. 1994; Chandler 1996; Dubocovsky et al. 1998; Michaels and Amasino 1999; Johanson et al. 2000; Kitahara and Matsumoto 2000; Kotoda et al. 2000; Sheldon et al. 2000; Gendall et al. 2001; Kitahara et al. 2001; Pelaz et al. 2001; Ampomah-Dwamena et al. 2002; Murai et al. 2003; Yan et al. 2003, 2004; Caicedo et al. 2004; Pillitteri et al 2004). These genes include those controlling vernalization and flower development. These genes are potential candidates for the rose everblooming gene (*evb*). To determine whether the genes are present at the rose cultivar, what relationship the genes with the *evb* gene have and further test the utility of the rose BAC library, we made two sets of hybond N+ filters of 7.5 x 11.5 cm from the library a '3x3' configuration. We also downloaded the DNA sequences of the genes cloned to date controlling vernalization and flower development from *Arabidopsis thaliana*, *Brassica rapa*, *Lycopersicon esculentum*, *Triticum monococcum*, and *Rosa rugosa* then designed primer pairs specific for each of the genes (Table 3). The library filters and gene-specific primer pairs have provided essential tools for isolation of the *evb* candidate genes from the rose everblooming cultivar Old Blush BAC library.

Table 3. Designed primers for isolation of homeologous and orthologous genes in rose using the rose cv. Old Blush BAC library.

Source species	Acc. No.	Genes	Forward (top) and reverse primers (5' – 3')	T_m	%GC	est.PCR Size(bp)
Arab. thaliana	AF116527	MADS box protein Flowering locus F (FLF)	**GTCGCTCTTCTCGTCGTCTC** **TACAAACGCTCGCCCTTATC**	60.3 59.8	60 50	570
	AY769360	Flowering locus C protein (FLC)	**TACCAACCTCTTTGGTACGG** **TTGAAAAGGCCACTGGAAAC**	57.6 60.1	50 45	2007
	AF284501	vernalization 2 protein (VRN2)	**AATTTAGGGGAGGCCTCAGA** **AAAAGCCGGAGATTTTCCTA**	60 58	50 40	1452
Brassica rapa	AY356368	reduced vernalization response 1 (VRN1)	AGGAAAGCTGACAACAACAACA **GGCAAATACATGATGCAACC**	59.8 58.9	41 45	593
Lyco. esculentum	AY306153	SEPALLATA3-like MADS-box (LeSEP3)	GGAGAGGTACCAGAAGTGCAA **CTAGGCCCTGCTCCTCCTAC**	59.3 60.4	52 65	492
Triticum monoc.	AY244506	MADS-box transcriptional factor (AGLG1)	**AGATGCAGGTGTGAGGCTTT** **TGGCAACTCTCTGTGTGTGA**	59.9 59	50 50	1061
	AY485974	ZCCT2 (VRN2)	**CCTCGGACCTTTGTGTTAGC** **AACCGCATGACATGGACATA**	59.7 59.8	55 45	481
	AB007504	TaMADS#11 (WAP1)	**CTGAAGGCGAAGGTTGAGAC** **TGCTTCTCGACGAGTTCCTT**	60 60.1	55 50	236
Rosa rugosa	AB055966	MASAKO B3	**TCAAGCAGAGACTACCAGGAA** CAGTCTAGCTTCCTTAACATAAATAGC	57.7 57.7	48 37	500
	AB038462	MASAKO BP	**GCGGATGAAAATCTTGGACA** **TGAATCAGTCAGGCACACCT**	61 59.3	45 50	600
	AB025645	MASAKO C2	**AGCAAGAAGCTGCCAAACTG** **ACCACAAAGCGAAGTTGGTT**	60.7 60	50 45	565
	AB099875	MASAKO euB3	**GAGATTCGCTGAACTGCACA** **GGAACGTCCAAAGATCAAGG**	60.1 59.5	50 50	566
	AB099876	MASAKO S1	CCCTTGAAAGGTACCAAAAATG **GCTGCAGTCATTTGCTGTGA**	59.8 61.2	41 50	499

DISCUSSION AND SUMMARY

Procedure of Megabase-sized DNA Preparation for Rose

We have developed a procedure for isolation of quality megabase-sized DNA from *Rosa* species. The DNA isolated with the procedure is not only large in size and readily digestible, but also readily clonable into large-insert cloning BAC vectors. Roses are among the species that are extremely abundant in phenolic compounds that bind DNA and inhibit isolation, making the DNA difficult to digest and clone. This is particularly true for large-insert DNA cloning, as encountered in our previous efforts in the rose species. Therefore, although procedures have been available for isolation of megabase-sized DNA from a variety of plant species (Zhang et al. 1995), it is essential to modify the current DNA isolation procedure or develop a new procedure for isolation of quality megabase-sized DNA from rose species. Zhao et al. (1994) and Kaufmann et al. (2003) attempted to control phenolics with PVP40 for preparation of megabase-sized DNA for cotton and rose, respectively. We also previously used the same chemical in preparation of megabase-sized DNA from cotton and rose (unpublished). The DNA isolated with the chemistry containing PVP40 was more readily digested than those without use of PVP40, but was not readily cloned into large-insert clones. The DNA plugs were dark brown in color, suggesting the existence of a significant amount of phenolics and the limited role of PVP40 in controlling phenolics. Instead of modifying the nuclei isolation buffer, we modified in this study the procedure of Zhang et al. (1995), including controlling the accumulation of phenolics in cytoplasm *in vivo* and extensively filtering the nuclei washes during nuclei isolation. Both approaches seemed helpful to reduce the phenolics contamination, nevertheless, increasing

the nuclei wash times is considered to be more efficient. This is because the treatment of plants in darkness was conducted in our previous studies using the buffer of Zhang et al. (1995) with or without PVP40 (unpublished), but the clonability of the resultant DNA was not improved significantly. The much lighter color of the DNA plugs prepared with the modified procedure and the results of DNA analysis and BAC library construction have proven the significant improvement of the DNA quality and feasibility of using the DNA for rose genome analysis and large-insert DNA cloning. The modified procedure of megabase-sized DNA isolation developed for rose may be applicable to many other plant species that are abundant in phenolics. These species include many of field crops such as cotton, ornamental plants such as roses, and trees such as pine and poplar. Differing from the chemical method such as use of PVP40 and other chemicals in the nuclei isolation buffer (Zhao et al. 1994; Kaufmann et al. 2003), the procedure reported in this study uses the same chemistry of Zhang et al. (1995) that has been widely used in preparation of megabase-sized DNA from a variety of plants. Simply increasing the nuclei wash times of the method is likely non-species-specific and thus, widely applicable to different species abundant in phenolics, whereas the efficiency of the chemical method on controlling phenolics may vary from species to species.

Large-insert BAC Library for Rose Everblooming Cultivar Old Blush

The successful development of a megabase-sized DNA isolation procedure for roses has allowed us to construct a BAC library from the DNA of the rose everblooming cultivar Old Blush. The library consists of 30,720 clones arrayed in 80 384-well microplates and has an average insert size of about 108 kb, thus covering 5.9 x rose genomes and providing a

greater than 99% probability of obtaining a clone from the library using any single-copy sequence as a probe. This *R. chinensis* library, along with the *R. rugosa* cv. Alba BAC library previously developed that has an average insert size of 103 kb and 5.2 x genome coverage (Kaufmann et al. 2003), collectively covers 11 x rose haploid genomes, therefore, the combined libraries will provide a comprehensive resource for genomics research of rose species. Moreover, as our library was constructed with *Bam*HI and that of Kaufmann et al. (2003) with *Hin*dIII, they are complementary to each other, thus providing a better genome coverage than the library constructed with a single enzyme (Wu et al. 2004; Ren et al. 2005). Finally, the *R. chinensis* library represents a different gene pool of roses from the *R. rugosa* library. Particularly, since the source of the *R. chinensis* library, cv. Old Blush, is everblooming (Semeniuk 1971; DeVries and Dubois 1978,1984), as is one of the parents of our population that is being used to genetically map the gene for everblooming (*evb*), the library will provide useful resources and tools to clone and characterize the *evb* gene.

Previous studies used double size selections for large-insert BAC library construction (Tao et al. 2002; Chang et al. 2003). In this study, we also conducted two size selections initially, but significantly smaller insert clones and lower transformation efficiency were obtained (data not shown). Therefore, a single round of size selection was performed to construct the rose cv. Old Blush BAC library. The construction of the rose cv. Old Blush BAC library indicates that one round size selection is sufficient for construction of BAC libraries having an average insert sizes of 100 – 140 kb (see Table 1). The smaller insert clones in the library resulted from the smaller fragments trapped in the larger fragments reduced the average insert size of the library, but may not significantly affect its utility for rose genome research.

The rose everblooming trait is a unique natural mutation in the plant kingdom controlled by a single gene. It not only converted the once blooming of the wild-type rose into recurrent blooming, but also significantly shorted the juvenile stage of plants. Therefore, isolation of the gene will provide essential tools and knowledge to modify the juvenile period and flowering nature in other ornamental and horticultural crops. Because there is no knowledge available about the biochemistry and nucleotide sequence of the *evb* gene, the candidate gene and positional cloning approaches have become the methods of choice to clone the *evb* gene. Several genes involved in plant flowering have been cloned from several plant species (Zemetra and Morris 1988; Schultz 1991; Mandel et al. 1992; Lee et al. 1994; Chandler 1996; Dubocovsky et al. 1998; Michaels and Amasino 1999; Johanson et al. 2000; Kitahara and Matsumoto 2000; Kotoda et al. 2000; Sheldon et al. 2000; Gendall et al. 2001; Kitahara et al. 2001; Pelaz et al. 2001; Ampomah-Dwamena et al. 2002; Murai et al. 2003; Caicedo et al. 2004; Pillitteri et al. 2004; Yan et al. 2003, 2004). Screening of the rose cv. Old Blush BAC library with the cloned gene sequences will not only facilitate characterization of the genes in roses, but also provide a great opportunity for identification and characterization of the *evb* gene due to cross homology of genes among related species.

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